

**SUBSTITUTE SPECIFICATION****TITLE****A METHOD FOR PRODUCING PARA-HYDROXYSTYRENE AND
OTHER MULTIFUNCTIONAL AROMATIC COMPOUNDS USING
TWO-PHASE EXTRACTIVE FERMENTATION**

This application claims the benefit of United States provisional applications 60/462,827, filed April 14, 2003 and 60/545888.60/_____, filed February 17, 2004, the disclosures of which are incorporated in their entirety by reference.

FIELD OF THE INVENTION

The invention relates to the field of bioprocessing. More specifically, the invention relates to a method for producing para-hydroxystyrene and other multifunctional aromatic compounds by microorganisms, in which the product is removed by extraction into an immiscible organic solvent during the fermentation.

BACKGROUND OF THE INVENTION

Multifunctional aromatic compounds, which comprise an aromatic ring and two or more functional groups, have potential utility in a wide variety of industrial applications. Examples include para-hydroxycinnamic acid (pHCA), cinnamic acid (CA), and para-hydroxystyrene (pHS). These aromatic compounds have application in monomers for the production of liquid crystalline polymers, and in the production of resins, elastomers, coatings, adhesives, automotive finishes and inks.

Chemical synthetic methods for producing these aromatic compounds are known. However, these chemical methods are expensive due to the high cost of the starting materials and the extensive product purification required. Moreover, these methods generate large amounts of unwanted byproducts. Consequently, biological production methods for these aromatic compounds have been developed. For example, Gatenby et al. in U.S. Patent No. 6,368,837 describe several methods for producing pHCA from glucose using recombinant microorganisms. A biological process for the production of pHS from a simple carbon source such as glucose is described by Ben-Bassat et al. in co pending U.S. Patent Application No. 60/383450. Additionally, Qi et al. in co pending U.S. Patent Application No. 10/138970 describe methods for producing CA and pHCA using recombinant microorganisms. However, a problem encountered with the biological production of these aromatic compounds is end-product inhibition, which limits product yield. Specifically, the rate

of production of the product by the microorganism decreases as the concentration of the product increases. Additionally, the microorganism becomes inactivated by the product when a certain critical concentration is reached in the fermentation medium.

5 One approach to mitigate end-product inhibition is to use two-phase extractive fermentation, in which the product is extracted into an immiscible organic phase during the fermentation so that it never reaches an inhibitory or critical concentration. In this way, the microorganism can function at a high rate of production over an extended period of time.

10 The principle of two-phase extractive fermentation was first described by Dreyfus in U.S. Patent No. 2,053,770. In that disclosure, the extractive fermentation of alcohol and acetone/butanol using organic solvents such as isoamyl alcohol is described. Kollerup et al. in U.S. Patent No. 4,865,973 and European Patent No. 0216221 describe a
15 process for the two-phase extractive fermentation of ethanol, acetone/butanol, penicillin, citric acid, and polysaccharides. In those disclosures a variety of solvents are taught, including: double bond
unsaturated aliphatic alcohols having 12 or more carbon atoms; saturated
20 branched chain aliphatic alcohols having 14 or more carbon atoms or mixtures thereof; double bond unsaturated aliphatic acids having 12 or more carbon atoms; aliphatic and aromatic mono-, di- or tri-esters having 12 or more carbon atoms, other than dibutyl phthalate; aliphatic noncyclic ketones and aliphatic aldehydes having 12 or more carbon atoms; and mixtures thereof.

25 There are other examples of two-phase extractive fermentation in the art. For example, see Jones et al., *Biotechnol. Lett.* 15:871-876 (1993); Daugulis et al., *Biotechnol. Lett.* 16:637-642 (1994); Lewis et al., *Biotechnol. Prog.* 8:104-110 (1992); and Barton et al., *Appl. Microbiol. Biotechnol.* 36:632-639 (1992). Lee et al. (*Enzyme Microb. Technol.* 23:261-266 (1998)) describe the production of 4-vinylguaiacol (4-hydroxy-3-methoxystyrene), a derivative of pHS, via the decarboxylation of ferulic acid by resting cells of *Bacillus pumilus* using a two-phase, biocatalytic process. Several solvents were evaluated, including chloroform,
30 methylene chloride, ethylacetate, ethyl ether, petroleum ether, cyclohexane, and C5-C8 alkanes. Hexane was selected as the preferred solvent. All of these solvents are toxic to microorganisms and/or are hazardous, and therefore, are unsuitable for the commercial scale production of multifunctional aromatic compounds via fermentation.

As indicated by the above, although methods have been developed for two-phase extraction of fermentation products, there have been no reports of the use of extractive fermentation in the production and recovery of pHCA, CA, or pHS. This may be in part because the development of novel two-phase extractive fermentation systems is a difficult and arduous task for one skilled in the art because of the unpredictable nature of these systems. For example, the selection of the solvent is very critical and the optimal solvent must be determined for each product/microorganism combination. The solvent must meet the following requirements for use in a commercial two-phase extractive fermentation process: low solubility in water, nontoxic to the producing microorganism, large partition coefficient for the product, low partition coefficient for nutrients, high selectivity, low emulsion forming tendency, high chemical and thermal stability, nonbiodegradability, nonhazardous, and low cost (Bruce et al., *Biotechnol. Prog.* 7:116-124 (1991)). Methods for predicting the biocompatibility of organic solvents (Bruce et al., *Supra*) have been suggested, however those methods address only one of the parameters needed for the development of a two-phase extractive solvent system. Moreover, those methods only serve as a guide for the selection of nontoxic solvents. The actual toxicity of the solvent can only be determined by experimental testing.

Toxicity testing of the solvent system is a particular problem and solvent toxicity is difficult to predict (Eiteman et al. *Appl. Microbiol. Biotechnol.* 30:614-618 (1989)). Playne et al. (*Biotechnol. Bioeng.* 25:1251-1265 (1983)) report diisoamyl ether (diisopentyl ether), is nontoxic toward anaerobic bacteria, as determined using flask tests. Staley et al. in WO 01/98521 describe a method for extracting carboxylic acids from fermentation broth using 2-decanone. Collins et al. (*Biotechnol. Bioeng.* 55:155-162 (1997)) and *Biotechnol. Techniques* 10:643-648 (1996)) describe the biodegradation of phenol by *Pseudomonas putida* in two-phase bioreactors using 2-undecanone as solvent. In that disclosure the organic solvent was used to control delivery of a toxic substrate, not to extract the product of a fermentation. Eiteman et al., *supra*, describe experimental testing of 24 solvents for two-phase extractive fermentation to produce 2,3-butanediol using *Klebsiella oxytoca*.

None of the above described solvents have been reported for the extraction of multifunctional aromatic compounds such as pHCA, CA, and

pHS, and clearly a need exists for fermentative extraction of these compounds.

Applicants have met the stated need by providing a method for the production of multifunctional aromatic compounds, such as pHCA, CA,
5 and pHs, in high yield using two-phase extractive fermentation with a novel group of extractive solvents.

SUMMARY OF THE INVENTION

The present invention provides methods for the recovery of multifunctional aromatic compounds from a fermentation medium via
10 solvent extraction. In one embodiment the invention provides a process for the recovery of a multifunctional aromatic compound from a fermentation medium comprising:

(a) providing a fermentation medium containing a multifunctional aromatic compound selected from the group consisting of cinnamic acid, para-hydroxycinnamic acid, and para-hydroxystyrene and mixtures
15 thereof;

(b) mixing the fermentation medium of (a) with an extractant selected from the group consisting of diisopentyl ether, n-propyl benzoate, 2-undecanone, dibenzyl ether, 2-tridecanone, 2-decanone, 1-phenyl-1-pentanone, methyl decanoate, 1-undecanol, diisobutyl DBE-IB and
20 mixtures thereof, for a time sufficient to allow extraction of the multifunctional aromatic compound into the extractant; and

(c) recovering the multifunctional aromatic compound from the extractant.

25 In an alternate embodiment the invention provides a process for the production of a multifunctional aromatic compound comprising:

(a) providing a production host which produces a multifunctional aromatic compound selected from the group consisting of cinnamic acid, para-hydroxycinnamic acid, and para-hydroxystyrene and mixtures
30 thereof;

(b) growing the production host in a fermentation medium wherein the production host produces a multifunctional aromatic compound into the fermentation medium to produce a conditioned medium;

(c) mixing the fermentation medium of (b) with an extractant
35 selected from the group consisting of diisopentyl ether, n-propyl benzoate, 2-undecanone, dibenzyl ether, 2-tridecanone, 2-decanone, 1-phenyl-1-pentanone, methyl decanoate, 1-undecanol, diisobutyl DBE-IB and

mixtures thereof, for a time sufficient to allow extraction of the multifunctional aromatic compound into the extractant;

(d) separating the extractant from the fermentation medium; and

(e) recovering the multifunctional aromatic compound from the
5 extractant.

In another preferred embodiment the invention provides a process for the production of a multifunctional aromatic compound comprising:

(a) providing a production host which produces a multifunctional aromatic compound selected from the group consisting of cinnamic acid,
10 para-hydroxycinnamic acid, and para-hydroxystyrene and mixtures thereof;

(b) growing the production host of step (a) in a biphasic growth medium comprising a fermentation medium containing from about 3% to about 60% by volume of an extractant, the extractant selected from the
15 group consisting of diisopentyl ether, n-propyl benzoate, 2-undecanone, dibenzyl ether, 2-tridecanone, 2-decanone, 1-phenyl-1-pentanone, methyl decanoate, 1-undecanol, diisobutyl DBE-IB and mixtures thereof; for a time sufficient to allow extraction of the multifunctional aromatic compound into the extractant;

(c) separating the extractant from the fermentation medium; and

(d) recovering the multifunctional aromatic compound from the
20 extractant.

BRIEF DESCRIPTION OF THE FIGURES, BIOLOGICAL DEPOSIT
AND SEQUENCE DESCRIPTIONS

25 The invention can be more fully understood from the following detailed description, figure, and the accompanying sequence descriptions, which form a part of this application.

Figure 1 is a graph of the growth of *E. coli* in the presence of various organic solvents plotted versus the log P value of the solvent.

30 Applicants made the following biological deposit under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure:

Depositor Identification	International Depository	
Reference	Designation	Date of Deposit
<i>E. coli</i> BNT511, tyrosine overproducing strain	ATCC: PTA-4314	May 9, 2002

The following sequences conform with 37 C.F.R. 1.821-1.825
("Requirements for Patent Applications Containing Nucleotide Sequences
and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are
5 consistent with World Intellectual Property Organization (WIPO) Standard
ST.25 (1998) and the sequence listing requirements of the EPO and PCT
(Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the
Administrative Instructions).

SEQ ID NO:1 is the nucleotide sequence of the para-
10 hydroxycinnamic acid decarboxylase gene (*pdh1*) from *Lactobacillus*
plantarum.

SEQ ID NOs:2 and 3 are nucleotide sequences of the primers used
to amplify *pdh1*, as described in Example 3.

SEQ ID NO:4 is the nucleotide sequence of the phenylalanine
15 ammonia lyase (*pal*) gene from *Rhodotorula glutinis*.

SEQ ID NOs:5 and 6 are nucleotide sequences of the primers used
to amplify *pal* from *Rhodotorula glutinis*, as describe in Example 3.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for the production and recovery of
20 multifunctional aromatic compounds of the invention by fermentation. The
method results in a higher rate of production of multifunctional aromatic
compound due to a decreased exposure of the product to the production
cells. The methods are useful as the multifunctional aromatic compounds
of the invention (para-hydroxycinnamic acid (pHCA), cinnamic acid (CA),
25 and para-hydroxystyrene (pHS)) have application as monomers for the
production of liquid crystalline polymers, and in the production of resins,
elastomers, coatings, adhesives, automotive finishes and inks.

The following definitions are used herein and should be referred to
for interpretation of the claims and the specification.

30 "CA" is the abbreviation used for cinnamic acid.

"pHCA" is the abbreviation used for para-hydroxycinnamic acid.

"pHS" is the abbreviation used for para-hydroxystyrene.

"TAL" is the abbreviation used for tyrosine ammonia lyase.

"PAL" is the abbreviation used for phenylalanine ammonia lyase.

35 "PAH" is the abbreviation used for phenylalanine hydroxylase.

"DBE" is the abbreviation used for diisobutyl DBE-IB, a product of
E. I. Du Pont de Nemours and Co. (Wilmington, DE). This product
consists of a mixture of diisobutyl succinate (20-30 wt%, CAS No. 925-06-

4), diisobutyl glutarate (55-70 wt%, CAS No. 71195-64-7), diisobutyl adipate (10-20 wt%, CAS No. 141-04-8), and traces of isobutanol (less than 0.25 wt%, CAS No. 78-83-1) and water (less than 0.15 wt%).

5 The term "TAL activity" refers to the ability of a protein to catalyze the direct conversion of tyrosine to pHCA.

The term "PAL activity" refers to the ability of a protein to catalyze the conversion of phenylalanine to cinnamic acid.

"*pal*" represents a gene that encodes an enzyme with PAL activity.

"*tal*" represents a gene that encodes an enzyme with TAL activity.

10 "*pdh*" represents a gene that encodes an enzyme with pHCA decarboxylase activity.

"*pdh1*" represents the gene that encodes an enzyme with pHCA decarboxylase activity from *Lactobacillus plantarum*.

15 The term "PAL/TAL activity" or "PAL/TAL enzyme" refers to a protein, which contains both PAL and TAL activity. Such protein has at least some specificity for both tyrosine and phenylalanine as an enzymatic substrate.

The term "P-450/P-450 reductase system" refers to a protein system responsible for the catalytic conversion of cinnamic acid to pHCA.
20 The P-450/P-450 reductase system is one of several enzymes or enzyme systems known in the art that performs a cinnamate 4-hydroxylase function. As used herein the term "cinnamate 4-hydroxylase" will refer to the general enzymatic activity that results in the conversion of cinnamic acid to pHCA, whereas the term "P-450/P-450 reductase system" will refer to a specific binary protein system that has cinnamate 4-hydroxylase
25 activity.

The "log P value" of a solvent refers to the logarithm of the solvent's partition coefficient in a standard octanol:water mixture. The log P value provides a quantitative measure of the polarity of the solvent.

30 The term "fermentable carbon substrate" refers to a carbon source capable of being metabolized by host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, one-carbon substrates and/or mixtures thereof.

35 The terms "growth medium", "fermentation medium" and "medium" are herein used interchangeably to refer to an aqueous solution containing nutrients for culturing microorganisms. The growth medium may additionally contain the microorganism, the product produced by the

microorganism, metabolic intermediates, and other components such as salts, vitamins, amino acids, cofactors, and antibiotics.

The term "biphasic growth medium" refers to a medium comprising a fermentation medium having a suitable amount of a water immiscible
5 extractant.

The term "extractant" refers to a solvent into which a multifunctional aromatic compound may be dissolved. Typical extractants of the invention are water-immiscible organic solvents.

"Nucleic acid" refers to a molecule, which can be single stranded or
10 double stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. In bacteria, lower eukaryotes, and in higher animals and plants, "deoxyribonucleic acid" (DNA) refers to the genetic material while "ribonucleic acid" (RNA) is involved in the translation of the information from DNA into proteins.

15 "Gene" refers to a nucleic acid fragment that effects the production of a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence.

"Transformation" refers to the transfer of a nucleic acid fragment
20 into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The term "expression" as used herein is intended to mean the
25 transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for the sequence of gene product is first transcribed to a complementary RNA which is often a messenger RNA and, then, the thus transcribed messenger RNA is translated into the above-mentioned gene product if
30 the gene product is a protein.

"Overproducing strain" refers to a recombinant microorganism that produces a gene product at a level that exceeds the level of production in normal or non-transformed microorganisms.

The term "production host" refers to a microorganism having the
35 ability to produce a multifunctional aromatic compound of the invention. As defined herein the production host may be a wildtype or recombinant variety.

The term "multifunctional aromatic compound" refers to organic compounds comprising an aromatic ring and two or more functional groups such as hydroxyl, vinyl, and carboxylic acid. The preferred multifunctional aromatic compounds of the invention include but are not limited to cinnamic acid (CA), para-hydroxycinnamic acid (pHCA), and para-hydroxystyrene (pHS).

The instant invention comprises a method for producing multifunctional aromatic compounds using two-phase extractive fermentation, in which a substrate is fermented in an aqueous medium by means of a microorganism and the resulting product is removed from the medium by extraction into an immiscible organic solvent during the fermentation.

Multifunctional Aromatic Compounds

The multifunctional aromatic compounds of the instant invention include, but are not limited to, cinnamic acid (CA), para-hydroxycinnamic acid (pHCA), and para-hydroxystyrene (pHS) and mixtures thereof. These aromatic compounds may be prepared by transformed prokaryotic systems, using standard recombinant DNA techniques. These recombinant DNA techniques are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), incorporated herein by reference. Suitable recombinant host cells include, but are not limited to *Escherichia*, *Methylosinus*, *Methylomonas*, *Pseudomonas*, *Streptomyces*, *Corynebacterium*, and *Rhodobacter*. These bacteria, particularly the Gram-negative organisms, are known to be tolerant of organic solvents (Inoue et al., *J. Ferment. Bioeng.* 71:194-196 (1991)). The preferred host cells of the instant invention are *Escherichia coli* and *Pseudomonas putida*. The most preferred host cells of the instant invention are mutant strains of these bacteria that overproduce either phenylalanine or tyrosine. Tyrosine overproducing strains are preferred when the production of pHS is carried out via a pathway that involves tyrosine, while phenylalanine overproducing strains are preferred when pHS is produced via a pathway that involves phenylalanine, as described *infra*. Tyrosine overproducing strains of *Escherichia* and *Pseudomonas*, as well as other bacteria, are known in the art (Maiti et al., *Antibiotic Bulletin* 37:51-65 (1995)). An example of an *Escherichia* tyrosine overproducing strain that may be used is *E. coli* TY1, available from OmniGene Bioproducts, Inc. Cambridge, MA. Phenylalanine

overproducing strains of *Escherichia* and *Pseudomonas*, as well as other bacteria, are also known in the art (Maiti et al, *supra* and Bongaertes et al., *Metabolic Engineering* 3:289-300 (2001)). An example of a phenylalanine overproducing strain that may be used is *E. coli* NST 74, available as

5 strain ATCC No. 31884 from the American Type Culture Collection, Manassas, VA. For example, pHCA and CA may be produced as described by Qi et al. in U.S. Patent Application Publication No. 2003-007925, incorporated herein by reference. According to that disclosure, CA may be produced using a recombinant microorganism engineered to

10 express at least one gene encoding a phenylalanine ammonia lyase (PAL) activity. This transformed microorganism metabolizes a fermentable carbon source, such as glucose, to phenylalanine, which is converted to CA by the PAL enzyme. That disclosure also teaches that pHCA may be produced using a recombinant microorganism engineered to express at

15 least one gene encoding a phenylalanine hydroxylase (PAH) activity and at least one gene encoding a tyrosine ammonia lyase (TAL) activity. This transformed microorganism metabolizes a fermentable carbon source, such as glucose, to phenylalanine, which is converted to tyrosine by PAH. The tyrosine produced is converted to pHCA by the TAL enzyme. Any

20 suitable enzyme possessing a TAL activity may be used. For example, an enzyme having both PAL and TAL (PAL/TAL) activity may be used. TAL enzymes, produced through mutagenesis of wild-type yeast PAL enzymes to have enhanced TAL activity, may also be used, as described by Gatenby et al. in U.S. Patent No. 6,368,837. Alternatively, an inducible

25 TAL enzyme from the yeast *Trichosporon cutaneum*, as described by Breinig et al. (U.S. Patent Application Publication No. 2004-002335) or a bacterial TAL enzyme such as that described by Kyndt et al. (FEBS Lett. 512:240-244 (2002)) or by Huang et al. (U.S. Patent Application Publication No. 2004-009795) may be used.

30 Para-hydroxycinnamic acid may also be produced by any one of the methods disclosed by Gatenby et al. *supra*, incorporated herein by reference. For example, pHCA may be produced using a recombinant microorganism engineered to express a gene encoding a yeast PAL activity and genes encoding a plant P-450/P-450 reductase system. This

35 transformed microorganism metabolizes a fermentable carbon source, such as glucose, to phenylalanine, which is converted to CA by the PAL enzyme. CA is subsequently converted to pHCA by the action of the P-450/P-450 reductase system. Alternatively, pHCA may be produced

using a recombinant microorganism expressing a gene encoding a TAL activity. The TAL enzyme converts tyrosine directly to pHCA. Any suitable TAL enzyme may be used, as described *supra*.

5 Para-hydroxystyrene may be produced as described by Ben-Bassat et al. in U.S. Patent Application Publication No. 2004-001860, incorporated herein by reference. In that disclosure, a biological method for producing pHS from a fermentable carbon source, such as glucose, is described. The transformed microorganism expresses at least one gene encoding a TAL activity and at least one gene encoding a pHCA
10 decarboxylase activity. The TAL enzyme converts tyrosine into pHCA, which is subsequently converted into pHS by the pHCA decarboxylase enzyme. Any suitable TAL enzyme described above may be used. In the preferred embodiment of the instant invention, pHS is produced by fermentation using a recombinant strain of *Escherichia coli* or
15 *Pseudomonas putida*.

Extractants

The extractants of the instant invention are water-immiscible organic solvents. The solvents were discovered to meet the criteria for an ideal solvent for a commercial two-phase extractive fermentation for the
20 production of multifunctional aromatic compounds. Specifically, these solvents were determined to be nontoxic to the preferred host cells, *Escherichia coli* and *Pseudomonas putida*, to be substantially immiscible with the fermentation medium, to have a high partition coefficient for the extraction of pHS and other multifunctional aromatic compounds, to have a
25 low partition coefficient for the extraction of nutrients, to have a low tendency to form emulsions with the fermentation medium, to be low cost and nonhazardous. The preferred solvents of the instant invention include: diisopentyl ether (CAS No. 544-01-4), n-propyl benzoate (CAS No. 2315-68-6), 2-undecanone (CAS No. 112-12-9), dibenzyl ether (CAS
30 No. 103-50-4), 2-tridecanone (CAS No. 593-08-8), 2-decanone (CAS No. 693-54-9), 1-phenyl-1-pentanone (CAS No. 1009-14-9), methyl decanoate (CAS No. 110-42-9), 1-undecanol (CAS No. 112-42-5), and DBE. The most preferred solvents of the instant invention are 2-undecanone, DBE, 2-tridecanone and methyl decanoate. Before use in a fermentation, these
35 solvents should be tested using shake-flask studies, as described in Example 2, *infra*, or agar plate studies, as described in Example 8, *infra*, to determine if they contain impurities that inhibit microbial growth. This was

a problem observed with the solvent 2-undecanone. Specifically, some lots of 2-undecanone were found to be toxic to *E. coli* due to impurities.

In some circumstances it may be advantageous to use a mixture of the preferred solvents. For example, solvent mixtures may be used to
5 increase the partition coefficient of the product. Additionally, solvent mixtures may be used to adjust and optimize physical characteristics of the solvent, such as the density, boiling point, and viscosity.

Under some conditions, the solvents of the instant invention, particularly DBE, may form a stable emulsion with the fermentation
10 medium. The stability of the emulsion formed is determined by the mode of fermentation used, the agitation and aeration rates, pH, temperature, the solvent, and the composition of the fermentation medium, especially the presence of colloidal insoluble solids, proteins, surface active agents, polymers, the amount of cells, and the presence of cell lysis material. The
15 formation of a stable emulsion complicates the separation of the fermentation medium from the organic extractant. The ease of separation of the two phases and the time required for the separation depends on the operational stability of the emulsion. If a stable emulsion is formed, it can be destabilized using methods known in the art (see for example, Mathys
20 et al. *J. Chem. Technol. Biotechnol.* 71:326-334 (1998), Schmid et al. *Enzyme and Microbial Technology* 22:487-493 (1998), Breen et al. *Surfactant Science Series* 61:237-285 (1996), and Isaacs et al. *Emulsions: Fundamentals and Applications in the Petroleum Industry*, Advances in Chemistry Series 231, L.L. Schramm, ed., American Chemical Society,
25 Washington, DC, pp 51-77 (1992)). For example, to minimize stable emulsion formation, the rate of agitation and aeration in the fermentor may be reduced. In addition, the pH and temperature of the fermentation medium may be adjusted to minimize the stability of the emulsion. The optimum conditions may be determined by routine experimentation.
30 Moreover, the amount of insolubles and polymers in the fermentation medium may be minimized by design of the medium or by filtration. The addition of surface-active agents may stabilize or destabilize the emulsion depending on the concentration used. The amount of surface-active agent required to destabilize the emulsion may be determined by routine
35 experimentation. Emulsion formation may also be minimized by reducing the contact time between the fermentation medium and the extractant. This may be accomplished by using the external extractive fermentation mode described *infra*. The formation of emulsions may further be

mitigated by separating the cells from the fermentation medium before contacting with the extractant in the external mode.

Methods for Producing Multifunctional Aromatic Compounds and Derivatives Thereof

5 To produce the multifunctional aromatic compound of interest, the microorganism to be used is cultured in a fermentor in a suitable growth medium. Any suitable fermentor may be used including a stirred tank fermentor, an airlift fermentor, a bubble fermentor, or any combination thereof. Materials and methods for the maintenance and growth of
10 microbial cultures are well known to those in the art of microbiology or fermentation science (See for example, Bailey et al., *Biochemical Engineering Fundamentals*, second edition, McGraw Hill, New York, 1986). Consideration must be given to appropriate growth medium, pH, temperature, and requirements for aerobic, microaerobic, or anaerobic
15 conditions, depending on the specific requirements of the microorganism, the fermentation, and the process. The growth medium used is not critical, but it must support growth of the microorganism used and promote the enzymatic pathway necessary to produce the desired product. A conventional growth medium may be used, including, but not limited to
20 complex media containing organic nitrogen sources such as yeast extract or peptone and a fermentable carbon source, minimal media, and defined media. Suitable fermentable carbon sources include, but are not limited to monosaccharides, such as glucose or fructose, disaccharides, such as lactose or sucrose, oligosaccharides and polysaccharides, such as starch
25 or cellulose, one-carbon substrates and/or mixtures thereof. In addition to the appropriate carbon source, the growth medium may contain a suitable nitrogen source, such as an ammonium salt, yeast extract or peptone, minerals, salts, cofactors, buffers and other components, known to those skilled in the art (Bailey et al. *supra*).

30 Suitable conditions for the extractive fermentation depend on the particular microorganism used and the product produced. Typical fermentation conditions are as follows. A suitable temperature for the fermentation ranges from about 20 °C to about 80 °C. The appropriate pH of the fermentation medium is from about 3.0 to about 9.0. For the
35 production of acidic compounds, such as CA and pHCA, a lower pH is preferred because the form of the product extracted into the organic phase is the unionized acid. Therefore, for acidic products the preferred pH range is from about 3.0 to about 6.0, depending on the ionization constant

(K_a) of the product and the growth of the microorganism at low pH. Suitable ranges for the pressure, the stir rate, and air-flow are 0.1 to 4.0 bar, 0 to 1500 rpm and 0 to 20 volume air per volume broth per min (VVM), respectively.

5 The solvent is typically added to the fermentation broth at the beginning of the fermentation. The volume of the organic solvent to be used depends on a number of factors, including the volume of the fermentation broth, the size of the fermentor, the partition coefficient of the solvent for the product, and the fermentation mode chosen, as described
10 *infra*. Typically the volume of the organic solvent is between about 3% to about 60% of the fermentor working volume.

 In a preferred embodiment of the invention, the two-phase extractive fermentation process is carried out in a continuous mode in a stirred tank fermentor. In this mode, a biphasic mixture consisting of the fermentation medium and the solvent containing the extracted product is
15 removed from the fermentor. The two phases are separated by means known in the art including, but not limited to, a gravity settler, a centrifuge and a hydrocyclone. An example of a gravity separator is described by Kollerup et al. in U.S. Patent No. 4,865,973, incorporated herein by
20 reference. The use of centrifuges and hydrocyclones is well known in the art of industrial processing (See for example, *Ullmann's Encyclopedia of Industrial Chemistry*, Fifth Edition, Elvers et al. Eds., VCH Publishers, New York, Vol. B2, Chapter 11, 1988). After separation, the fermentation medium is recycled to the fermentor and the solvent is treated to recover
25 the product by means described *infra*. The solvent may then be recycled back into the fermentor for the further extraction of the product. Alternatively, fresh solvent may be continuously added to the fermentor to replace the removed solvent. This continuous mode of operation offers several advantages. Because the product is continually removed from the
30 reactor, a smaller volume of organic solvent is required, thus enabling a larger volume of the fermentation broth to be used. This results in higher production yields. In this mode, the volume of the organic solvent is between about 3% to about 30%, preferably between about 3% to about 20%, most preferably between about 3% to about 10% of the fermentor
35 working volume. It is preferred to use the smallest amount of solvent in the fermentor as possible to maximize the volume of the aqueous phase, and therefore, the amount of cells in the fermentor. Moreover, the process may be operated in an entirely continuous mode in which the solvent is

continuously recycled between the fermentor and a separation apparatus and the fermentation medium is continuously removed from the fermentor and replenished with fresh medium. In this entirely continuous mode, the product is not allowed to reach the critical toxic concentration and fresh
5 nutrients are continuously provided so that the fermentation may be carried out for long periods of time. The apparatus that may be used to carryout these modes of two-phase extractive fermentations are well known in the art. Examples are described by Kollerup et al. in U.S. Patent No. 4,865,973, incorporated herein by reference.

10 In another embodiment of the instant invention, a batchwise fermentation mode is used. Batch fermentation, which is well known in the art, is a closed system in which the composition of the medium is set at the beginning of the fermentation and is not subjected to artificial alterations during the process. In this mode, a volume of organic solvent is added to
15 the fermentor at the beginning of the fermentation and the solvent is not removed during the process. Although this mode is simpler than the continuous or the entirely continuous modes described *supra*, it requires a larger volume of organic solvent to minimize the concentration of the inhibitory product in the growth medium. Consequently, the volume of the growth medium must be less and the amount of product produced is less
20 than that obtained using the continuous mode. In the batchwise mode, the volume of the organic solvent is between about 20% to about 60%, preferably between about 30% to about 60% of the fermentor working volume. It is preferred to use the smallest volume of solvent in the
25 fermentor as possible, for the reason described *supra*.

In another embodiment of the instant invention, a fed-batch fermentation mode is used. Fed-batch fermentation is a variation of the standard batch system, in which the nutrients, for example glucose, are added in increments during the fermentation. The amount and the rate of
30 addition of the nutrient may be determined by routine experimentation. For example, the concentration of critical nutrients in the fermentation broth may be monitored during the fermentation. Alternatively, more easily measured factors such as pH, dissolved oxygen, and the partial pressure of waste gases, such as carbon dioxide, may be monitored. From these
35 measured parameters, the rate of nutrient addition may be determined. The amount of organic solvent used in this mode is the same as that used in the batch-wise mode, described *supra*.

In yet another embodiment of the instant invention, the extraction of the product may be done downstream of the fermentor, rather than *in situ*. In this external mode, a batch-wise fermentation or a fed-batch fermentation is used and the liquid-liquid extraction of the product is

5 carried out on the fermentation medium, removed from the fermentor. The amount of organic solvent used in this mode is between about 20% to about 60%, preferably between about 30% to about 60% of the fermentor working volume. The fermentation medium may be removed from the fermentor continuously or periodically, and the extraction of the product by

10 the organic solvent may be done with or without the removal of the cells from the fermentation medium. The cells may be removed from the fermentation medium by means known in the art including, but not limited to, filtration or centrifugation. After separation of the fermentation medium from the solvent by means described *supra*, the fermentation medium may

15 be recycled into the fermentor, discarded, or treated for the removal of any remaining product. Similarly, the isolated cells may also be recycled into the fermentor. After treatment to recover the product, the solvent may be recycled for use in the extraction process. Alternatively, fresh solvent may be used. In this mode the solvent is not present in the fermentor, so the

20 toxicity of the solvent is much less of a problem. If the cells are separated from the fermentation medium before contacting with the solvent, the problem of solvent toxicity is further reduced. Furthermore, using this external mode there is less chance of forming an emulsion and evaporation of the solvent is minimized, alleviating environmental

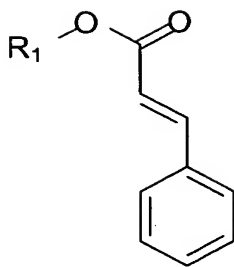
25 concerns.

After the product is extracted from the fermentation medium into the solvent, it may be recovered from the solvent by methods well known in the art, including but not limited to distillation, adsorption by resins or separation by molecular sieves. The preferred method of product recovery

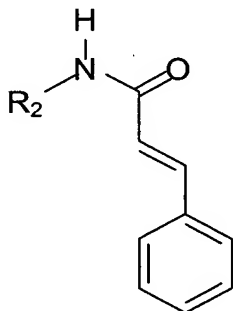
30 from the organic solvent is distillation.

Additionally, the product may be chemically derivatized in the extractant before it is recovered. Any suitable chemical derivatization method known in the art may be used. For example, CA derivatives include, but are not limited to, esters, amides, aldehydes, ketones and

35 anhydrides. Suitable CA derived esters include, but are not limited to, compounds having the general formula given by:



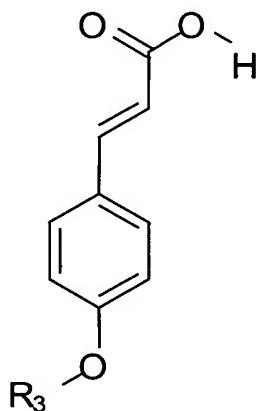
wherein R₁ is allyl (Takeda et al., *Tetrahedron Lett.* 36:113 (1995)), methyl (Parrish et al., *Synth. Commun.* 30:2687 (2000)), ethyl (Kumar et al., *Synth. Commun.* 14:1359 (1984)), n-butyl or other straight chain alkyl groups (Cablewski et al., WO 9959947), t-butyl (Wright et al., *Tetrahedron Lett.* 38:7345-7348 (1997)), benzyl (Ahmed et al., *Chem. Lett.* 1980, 1161), phenyl (Womack et al., *J. Org. Synth.* III:714 (1955)), hydroxyethyl (Mikhant'eva et al., *Zhurnal Prikladnoi Khimii* 54:1203-1206 (1981)), vinyl (Ishihara et al., *J. Mol. Cat. B: Enzymatic* 7:307-310 (1999)), methoxyethyl (Vora et al., *Ind. Acad. Sci. Chem. Sci* 113:95-102 (2001)), or glycidyl (Nair et al., *Synth. Commun.* 29:2559-2566 1999)). The references given in parentheses, all of which are incorporated herein by reference, describe methods that may be used to synthesize the cited derivative. CA derived amides include, but are not limited to, compounds having the general formula given by:



wherein R₂ is H (Pellegata et al., *Synthesis* 1985:517), NH₂ (Lifka et al., *J. fuer Praktische Chemie/Chemiker-Zeitung* 337:641-646 (1995)), benzyl (Hossain et al., *Ind. J. Chem. B* 29:1062-1063 (1990)), cyclohexyl (Ueda et al., *Bull. Chem. Soc. Jpn.* 65:1636-1641 (1992)), or phenethyl (Kunishima et al., *Tetrahedron Lett.* 40:5327-5330 (1999)). Other CA derivatives include, but are not limited to, cinnamic anhydride (Arrieta et al., *Synth. Commun.* 13:471 (1983)), cinnamaldehyde (Cha et al., *Org. Prep. Proced.*

Int. 31:694-697 (1999)), the phenyl ketone of CA (Alonso et al., *J. Org. Chem.* 61:6058-6059 (1996)), the epoxide of the cinnamyl double bond (Rozen et al., *Tetrahedron Lett.* 37:531-534 (1996)), reduction of cinnamate derivatives (Elamin et al., *Tetrahedron Lett.* 29:5599 (1988))
5 and esters of poly(vinyl alcohol) (Chetri et al., *J. Polym. Sci. Pol. Chem.* 34:1613-1615 (1996)). The references cited in parentheses, all of which are incorporated herein by reference, describe methods that may be used to synthesize the cited derivative.

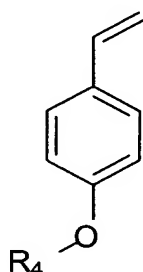
Suitable pHCA derivatives include those formed by chemical
10 modification of the hydroxyl group, the carboxylate group or the cinnamyl double bond. Chemical derivatives formed by the modification of the carboxylate group or double bond include, but are not limited to, the same derivatives that are described for CA, *supra*. Derivatives of the pHCA hydroxyl group include, but are not limited to, compounds having the
15 general formula given by:



wherein R₃ is acetoxy (Hosoda et al., *J. Org. Chem.* 66:7199-7201 (2001)), sulfonic acid (Alexandratos et al., U.S. Patent No. 5,990,336)), a
20 long chain alkyl (Watanabe et al., *J. Med. Chem.* 23:50-59 (1980)), ethoxycarbonyl (Kellard et al., *Colloque Scientifique International sur le café* 12:254-259 (1988)), trimethylsilyl (Ford et al., *J. Chromatogr.* 436:484-489 (1988)), t-butyldimethylsilyl, diethyl phosphonate, or phosphate (Szardenings et al., *Tetrahedron Lett.* 37:3635-3638 (1996)).
25 Additionally, the derivative may be a dibenzyl derivative wherein R₃ is benzyl and the carboxylate hydrogen is replaced with a benzyl group (Waid et al., *Tetrahedron Lett.* 37:4091-4094 (1996)) or a glycidyl ether derivative wherein R₃ is glycidyl and the carboxylate hydrogen is replaced

with a methyl group (Erhardt et al., *J. Med. Chem.* 25:1408-1412 (1982)). The pHCA product may also be decarboxylated to pHS using the method described by Cohen et al. (*J. Amer. Chem. Soc.* 82:1907-1911 (1960)). The references cited in parentheses, all of which are incorporated herein
5 by reference, describe methods that may be used to synthesize the cited derivative.

Suitable derivatives of pHS include, but are not limited to, various ethers and esters. Examples of ethers and esters derived from pHS
10 include, but are not limited to, compounds having the general formula given by:



wherein R_4 is methyl (Hattori et al., *J. Amer. Chem. Soc.* 81:4424-4427 (1959)), t-butyl (Gable et al., *J. Amer. Chem. Soc.* 124:3970-3979 (2002)),
15 alkyl (Hassanein et al., *J. Org. Chem.* 54:3106-3113 (1989)), silyl ethers (Nakahama et al., *Prog. Polym. Sci.* 15:299-335 (1990)), allyl (Woods et al., U.S. Patent No. 5,633,411), t-butoxy carbonyl (Nader et al., U.S. Patent No. 5,082,965), hydroxyethoxy (Inokuma et al., *Heterocycles* 40:401-411 (1995)), acetoxymethyl (Sounik et al., U.S. Patent No. 5,463,108),
20 formate (Tessier et al., *Materials for Microlithography: Radiation-Sensitive Polymers*, ACS Symposium Series 266, American Chemical Society, Washington, DC, 1984), glycidyl (Ericsson et al., U.S. Patent No. 6,255,385), benzoate (Hattori et al., *J. Amer. Chem. Soc.* 81:4424-4427 (1959)), phenylcarbonate (Whitcombe et al., *J. Amer.*
25 *Chem. Soc.* 117:7105-7111), tetrahydropyran (Menzler et al., *Bioorg. Med. Chem. Lett.* 10:345-348 (2000)), benzyl (Kotecha et al., *Synlett* 1992:395), or poly(ethylene oxide) (Inokuma et al., *Heterocycles* 54:123-130 (2001)). The references cited in parentheses, all of which are incorporated herein by reference, describe methods that may be used to
30 synthesize the cited derivative. Additionally, pHS may be polymerized to poly(para-hydroxystyrene) and other copolymers using the methods

described by Kaneko et al. in U.S. Patent Nos. 5,959,051 and 6,258,901, both of which are incorporated herein by reference. The preferred pH derivative of the instant invention is para-acetoxystyrene, which may be prepared using the method described by Sounik et al. in U.S. Patent
5 No. 5,463,108. Following derivatization, the derivatized product is recovered from the organic solvent using the methods described *supra*.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred
10 embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and
15 conditions.

Unless otherwise specified, all chemicals used in the following Examples were reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO).

The meaning of abbreviations used is as follows: "min" means
20 minute(s), "h" means hour(s), "rpm" means revolutions per minute, "μL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "nm" means nanometer(s), "mm" means millimeter(s), "cm" means centimeter(s), "μm" means micrometer(s), "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "μmole" means micromole(s),
25 "g" means gram(s), "μg" means microgram(s), "mg" means milligram(s), "g" means the gravitation constant, "OD" means optical density, "OD550" means the optical density at a wavelength of 550 nm, "OD600" means the optical density at a wavelength of 600 nm, "HPLC" means high performance liquid chromatography, "Aq" means aqueous phase, "Org"
30 means organic phase, "IPTG" means isopropyl β-D-thiogalactopyranoside, and "SLPM" means standard liter per minute, "kV" means kilovolt(s), and "μF" means microfarad(s).

General Methods:

HPLC Determination of Tyrosine, Phenylalanine, CA, pHCA, and pHs:

35 Samples were analyzed for tyrosine, phenylalanine, CA, pHCA, and pHs using reverse-phase chromatography with an Agilent HP1100 HPLC system equipped with an autosampler, diode-array detector, and ChemStation for data acquisition and processing (Agilent Technologies,

Inc., Wilmington, DE). The analyses were done using a 4.6 mm x 15 cm Zorbax SB-C18 column (3.5 μ m particle size), obtained from Agilent Technologies, Inc., thermostated at 35 °C. The samples were run using a flow rate of 1.0 mL/min with an eight-minute solvent gradient from 5% acetonitrile/water, containing 0.1% trifluoroacetic acid, to 80% acetonitrile/water, containing 0.1% trifluoroacetic acid. A diode-array detector set at 210, 260, and 280 nm was used to detect the various compounds at their optimum absorbance wavelength.

The aqueous samples were diluted 1:3 with water, while the organic samples were diluted 1:3 with acetonitrile before injection of 50 μ L of the sample into the HPLC. A standard mixture of the analytes was prepared by mixing equal portions of solutions of the individual components, each at a concentration of 1 mg/mL.

EXAMPLE 1

The Effect of pHCA and pHS on the Growth of *E. coli*

The purpose of this Example was to determine the toxicity of pHCA and pHS toward *E. coli*.

This study was done using *E. coli* TY1 (DGL430), a tyrosine overproducing strain obtained from OmniGene Bioproducts, Inc. (Cambridge, MA). This strain also produces phenylalanine. The seed culture of this strain was propagated in a medium consisting of MgSO₄·7H₂O (0.5 g/L), (NH₄)₂SO₄ (4 g/L), MOPS (20.9 g/L) and 10 mL of phosphate solution. The pH of the seed medium was adjusted to pH 6.8 and then the medium was steam-sterilized. The phosphate solution contained KH₂PO₄ (57.3 g/L) and K₂HPO₄ (110.2 g/L) and was adjusted to pH 6.8. The following solutions were then aseptically added to the seed medium to give the final concentration given in parentheses: thiamine (1 mg/L), glucose (1 g/L), lactose (15 g/L), 5 mL of a trace element solution, and tetracycline (5 mg/L). The trace element solution contained citric acid (10 g/L), CaCl₂·2H₂O (1.5 g/L), FeSO₄·7H₂O (5 g/L), ZnSO₄·7H₂O (0.39 g/L) and CuSO₄·5H₂O (0.38 g/L). The seed medium was inoculated from a frozen pre-seed culture and was grown in 250 mL baffled flasks in an Innova 4000 incubator shaker (New Brunswick Scientific Co., Edison, NJ), incubated at 35 °C and 300 rpm for 12-16 h. One milliliter of this seed culture was used to inoculate each of the test cultures.

The test medium was 50 mL of an aqueous solution containing: (NH₄)₂SO₄ (4 g), 1 mL of phosphate solution, MgSO₄·7H₂O (0.5 g), and

MOPS (20.9 g), with the pH adjusted to 7.0. After sterilization of this medium, thiamine (1 mg/L), 5 mL of trace element solution, glucose (2 g/L), lactose (15 g/L), IPTG (0.5 mM), and tetracycline (10 mg/L) were aseptically added to give the final concentration given in parentheses.

- 5 Sodium pHCA was added to the culture flasks as shown in Table 1. The 250 mL baffled flasks containing 50 mL of the test medium were inoculated with the seed culture and were incubated at 35 °C and 300 rpm for 42 h in the incubator shaker. Samples of the culture were taken at various times and the optical density at 550 nm (OD550) was measured
- 10 spectrophotometrically. The pH of these samples was also measured. After completion of the fermentation, the tyrosine and phenylalanine concentrations in the culture were measured using HPLC, as described *supra*. As can be seen from the results given in Table 1, pHCA inhibited
- 15 inhibition of growth was observed at a pHCA concentration of 8 g/L.

Table 1
The Effect of pHCA on Growth and Fermentation of *E. coli*

20

	0 h	17.5 h		29 h		42 h			
pHCA added, g/L	OD 550	OD550	pH	OD 550	pH	OD 550	pH	Tyr mg/L	Phe mg/L
0.00	~0.05	1.14	6.75	5.44	6.13	5.52	6.17	700	200
1.00	~0.05	2.41	5.87	3.57	5.30	3.48	5.23	190	155
2.00	~0.05	1.27	5.97	1.47	5.68	1.53	5.58	105	95
4.00	~0.05	0.20	6.79	1.21	6.52	1.54	6.10	65	15
8.00	~0.05	0.15	6.84	0.16	6.82	0.18	6.79	15	10
16.00	~0.05	0.19	6.98	0.21	6.99	0.23	7.01	0	5
0.00	~0.05	1.19	6.65	6.49	6.15	5.20	6.17	680	200
1.00	~0.05	2.47	5.89	3.22	5.36	3.37	5.23	210	150
2.00	~0.05	1.49	5.89	1.77	5.64	1.62	5.54	100	95
4.00	~0.05	0.22	6.81	1.61	6.43	1.55	6.03	75	20
8.00	~0.05	0.15	6.84	0.16	6.81	0.17	6.78	25	10
16.00	~0.05	0.13	6.97	0.13	7.00	0.14	7.00	0	5

This experiment was repeated using pHS in place of pHCA. Concentrations of pHS of 0.2 g/L inhibited the growth of *E. coli* and no

growth was observed at a concentration of 0.4 g/L. These results demonstrate that both pHCA and pHS inhibit the growth of *E. coli*, thereby limiting the yield of these products.

EXAMPLE 2

5 Effect of Organic Solvents on the Growth of *E. coli*

The purpose of this Example was to investigate the effect of the organic solvents 1-undecanol and 2-undecanone on the growth of *E. coli*.

10 *E. coli* strain BNT511 was used in this study. This strain is a tyrosine overproducing strain derived from *E. coli* strain NST 74 (ATCC No. 31884), which was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Strain BNT511 is deposited with the American Type Culture Collection under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and has been assigned a depository
15 designation of PTA-4314. *E. coli* strain BNT511 was obtained as follows. A culture of *E. coli* strain NST 74 was grown overnight at 37 °C in three milliliters of M9 medium, prepared as follows. A solution of M9 salts buffer consisting of KH₂PO₄ (3 g/L), Na₂HPO₄ (6 g/L), NaCl (0.5 g/L), and NH₄Cl (1 g/L), was prepared and the pH was adjusted to 7.4. This M9
20 salts buffer was autoclaved and then the following solutions, each sterilized separately by filtration (glucose) or autoclaving, were added to give the concentrations given in parentheses MgSO₄ (0.24 g/L), glucose (0.2%), CaCl₂ (0.011 g/L), and yeast extract (1 g/L). After growth, the culture was diluted by a factor of 10³ to 10⁶ in M9 salts buffer. Then,
25 100 µL of each dilution was plated on M9+1% glucose agar containing 0, 50, 150, or 450 mg/mL of 3-fluorotyrosine and the plates were incubated at 37 °C. After 18 h, growth was observed only on the plate not containing 3-fluorotyrosine. Plates containing 3-fluorotyrosine were incubated an additional 24 h, after which time, several isolates were identified. One
30 NST 74-derived isolate was obtained from each of the 150 and 450 mg/mL 3-fluorotyrosine plates. Individual isolates were propagated on M9+1% glucose agar containing 0, 50, 150, or 250 mg/mL 3-fluorotyrosine. One of these two isolates was designated as BNT511.

35 *E. coli* strain BNT511 was grown in a medium containing KH₂PO₄ (0.15 g/L), Na₂HPO₄ (0.35 g/L), (NH₄)₂ SO₄ (3 g/L), MgSO₄·7H₂O (0.3 g/L), yeast extract (0.5 g/L), MOPS (15.6 g/L), and glucose (10 g/L), adjusted to pH 6.8. A seed culture, taken from an active culture that grew in a fermentor and was diluted to 0.3 OD at 550 nm, was used to inoculate

30 mL of culture medium in 250 mL baffled flasks. Ten milliliters of the solvent to be tested, either 1-undecanol (98%, obtained from Aldrich) or 2-undecanone (98%, obtained from Aldrich), was added to the flask and the flasks were incubated at 35 °C and 300 rpm for 22 h in an incubator shaker. A flask without an organic solvent added served as the control. This experiment was run in duplicate. Samples of the aqueous culture media were removed at various times and the pH and the OD550 were measured. As shown by the results given in Table 2, the presence of the organic solvents tested had no significant effect on the growth of *E. coli*. However, the results obtained with 2-undecanone in these studies were not consistent. Specifically, 2-undecanone from some suppliers and even from different lots from the same supplier were found to inhibit growth. We believe that this variability was caused by variations in the purity of this solvent.

Table 2
Effect of Organic Solvents on the Growth of *E. coli*

Time, h	Control, no solvent				With 1-undecanol				With 2-undecanone			
	OD550	pH	OD550	pH	OD550	pH	OD550	pH	OD550	pH	OD550	pH
0	0.3	6.8	0.3	6.8	0.3	6.8	0.3	6.8	0.3	6.8	nt ¹	6.8
2	1.012	7.06	0.96	nt	1.428	7.12	1.456	nt	1.004	7.3	1.004	nt
4.2	1.78	6.83	1.82	6.77	1.96	6.76	2.14	6.75	1.88	6.7	1.88	6.7
6	6.48	nt	6.68	nt	6.02	nt	6.7	nt	6.28	nt	6.3	nt
22	14.1	6.64	13.6	6.65	19.1	6.71	15.7	6.7	12.3	6.61	15.2	6.6

¹nt means not tested

EXAMPLE 3

Production of pHS in a Two-Phase Extractive Fermentation System

The purpose of this Example was to demonstrate the production of pHS in a two-phase extractive fermentation system and to compare the yield with that obtained in a single phase fermentation.

The strain used in this study was *E. coli* WWQ51.1, which was designed for the production of pHCA and pHS from glucose, as described in co pending U.S. Patent Application Publication No. 2004-001860. This strain also produces CA, phenylalanine, and tyrosine, and was derived by transforming *E. coli* NST 74 with the phenylalanine ammonia lyase gene

(*pal*) from *Rhodospiridium glutinis* and the para-hydroxycinnamic acid decarboxylase gene (*pdcl*) from *Lactobacillus plantarum*.

Construction of *E. coli* WWQ51.1

The *pdcl* gene (GenBank Accession No. U63827), given as SEQ ID NO:1, was amplified by PCR using genomic DNA from *Lactobacillus plantarum* as template. The genomic DNA was isolated from *Lactobacillus plantarum* (ATCC No. 14917) grown on MRS medium (ATCC Medium No. 416) using a DNeasy[®] Kit (Qiagen, Valencia, CA). The oligonucleotide primers used to amplify the *pdcl* gene were 5'-GGTAATTCATATGACAAA-3', given as SEQ ID NO:2, and 5'-TCACGTGAAACATTACTTATT-3', given as SEQ ID NO:3, which included an *NdeI* site (underlined nucleotides). The expected 550-bp DNA fragment was purified using a Qiagen PCR Clean Up Kit and was ligated into the pCRII-TOPO cloning vector using the TA Cloning[®] Kit from Invitrogen (Carlsbad, CA). Subsequently, this plasmid was digested with *Bam*HI and *Xba*I and the fragments containing the *pdcl* gene were ligated into pKSM715 (obtained from ATCC), which had been previously digested with *Bam*HI and *Xba*I, to form pKSM-*pdcl*.

The *Rhodotorula glutinis* (ATCC No. 10788) *pal* gene, SEQ ID NO:4 (GenBank Accession No. M18261), was amplified from reverse-transcribed RNA that was purified from exponential phase cells grown in YM broth (ATCC Medium No. 200), supplemented with phenylalanine. The *Rhodotorula glutinis* mRNA was reversed transcribed according to the Perkin Elmer (Norwich, CT) GeneAmp Kit instructions without diethylpyrocarbonate (DEPC) treated water. The primers used were the random hexamers supplied with the kit. Primers used to amplify the *pal* gene included the upstream primer 5'-ATAGTAGAATTCATGGCACCTCGCTCGACTCGA-3' (SEQ ID NO:5) containing an *Eco*RI restriction site, and a downstream PCR primer 5'-GAGAGACTGCAGAGAGGCAGCCAAGAACG-3' (SEQ ID NO:6) containing a *Pst*I restriction site which were synthesized based on the *Rhodospiridium toruloides pal* gene. PCR fragments were digested with *Eco*RI and *Pst*I and ligated to pKK223-3 (Amersham Biosciences, Piscataway, NY), previously cut with *Eco*RI and *Pst*I, forming pCA16.

Electro-competent *E. coli* strain NST 74 cells were prepared as follows. Samples of glycerol stocks of *E. coli* strain NST 74 were spread onto LB agar plates without any antibiotics and incubated overnight at 37 °C. A single colony was picked, inoculated into 4.0 mL of LB medium

(ATCC Medium No. 1065) and grown overnight at 37 °C. One liter of LB medium was inoculated with a 1/100 volume of the fresh overnight culture. The cells were grown at 37 °C, with vigorous shaking, to an OD of approximately 0.5 to 0.7 at 600 nm. The cells were centrifuged in cold
5 centrifuge bottles in a cold rotor at 4000 x g for 15 min. The supernatants were discarded and the cell pellets were gently resuspended in a total of 1.0 L of ice-cold 10 % glycerol. The cells were centrifuged as described above and then resuspended in 0.5 L of ice-cold 10% glycerol. The above procedures were repeated to obtain the cells, which were resuspended in
10 250 mL of ice-cold 10 % glycerol and washed again. The cell pellets obtained from the last washing were resuspended into a final volume of 3.0 - 4.0 mL in ice-cold 10% glycerol. The cell suspension was frozen in aliquots on dry ice, and stored at -80 °C.

The electro-competent *E. coli* NST 74 cells were thawed on ice and
15 the plasmids pKSM-pdc1 and pCA16 (~50 ng each), described above, were added to the tubes containing the cells. The DNA/cell suspensions were then transferred to pre-chilled electroporation cuvettes (0.1 cm, Bio-Rad, Hercules, CA) and the cuvettes were kept on ice. Each sample was electrically pulsed at 18 kV/cm in a Gene Pulser (Bio-Rad) (25 µF, 200
20 ohm). SOC medium (1.0 mL, available from Invitrogen) was added to each cuvette immediately after pulsing. The cell mixtures were then transferred to tubes and left on the shaker (1.0 h at 37 °C, and shaken at 220 rpm). Samples (100 µL) of each transformation reaction were then pipetted onto separate LB plates, containing 100 µg/mL ampicillin and 50
25 µg/mL kanamycin, and incubated overnight at 37 °C. Single colonies were selected and grown in LB medium containing the antibiotics overnight to give the seed culture.

Production of pHS

The seed culture was propagated in 250 mL baffled flasks,
30 incubated at 35 °C and 300 rpm in an incubator shaker, in a medium consisting of MgSO₄·7H₂O (0.5 g/L), (NH₄)₂SO₄ (4 g/L), MOPS (20.9 g/L) and 10 mL of phosphate solution (as described in Example 1). The pH of the seed medium was adjusted to pH 6.8 and it was steam-sterilized. The following solutions were then aseptically added to the seed medium to give
35 the final concentration given in parentheses: thiamine (1 mg/L), glucose (15 g/L), 5 mL of a trace element solution (as described in Example 1), kanamycin (50 mg/L) and ampicillin (100 mg/L).

The seed medium was inoculated from a frozen pre-seed culture and was grown in a flask for 12-16 h. One milliliter of this seed culture was used to inoculate each of the test cultures. The test medium was 50 mL of an aqueous solution containing (NH₄)₂SO₄ (4 g), 1 mL of phosphate solution (as described in Example 1), MgSO₄·7H₂O (0.5 g) MOPS (20.9 g) with the pH adjusted to 7.0. After sterilization of this medium, thiamine (1 mg/L), 5 mL of trace element solution (as described in Example 1), glucose (15 g/L), IPTG (0.5 mM), kanamycin (50 mg/L) and ampicillin (100 mg/L) were aseptically added to give the final concentration given in parentheses. Four test cultures were inoculated; two contained 20 mL of 2-undecanone, the other two contained no organic solvent and served as controls. The cultures were incubated in 250 mL baffled flasks in an incubator shaker at 35 °C and 300 rpm.

A sample of the aqueous phase was withdrawn at various times and the OD550, the pH, the glucose concentration, and the pHS concentration were measured. The organic phase was also sampled and the concentration of pHS was determined. The pHS concentrations were determined using HPLC, as described *supra*. The glucose concentration was measured either using a YSI Glucose Analyzer (YSI Inc., Yellow Springs, OH) or using a glucose assay kit obtained from Sigma Chemical Co. (St Louis, MO).

As can be seen from the results shown in Table 3, most of the pHS produced was extracted into the organic phase and the pHS production (total mg) in the two-phase system was approximately 2.5-fold higher than that obtained in the conventional single phase system.

Table 3
Comparison of the Production of pHS in Two-Phase and
Single Phase Fermentations

0 h results				
Flask No.	OD550	Glucose g/L	pHS Aq mg/L	pHS Org mg/L
1 - 1 phase	0.12	17.5	0.009	na ²
2 - 1 phase	0.12	17.7	0.009	na
3 - 2 phase	0.12	17.6	nt ¹	nt
4 - 2 phase	0.12	17.6	nt	nt

9 h results				
Flask No.	OD550	Glucose g/L	pHS Aq mg/L	pHS Org mg/L
1 - 1 phase	6.8	11.4	3.76	na
2 - 1 phase	7.2	12.4	2.59	na
3 - 2 phase	nt	nt	nt	nt
4 - 2 phase	nt	nt	nt	nt
25 h results				
Flask No.	OD550	Glucose g/L	pHS Aq mg/L	pHS Org mg/L
1 - 1 phase	10.2	4.73	15.29	na
2 - 1 phase	9.9	4.53	9.88	na
3 - 2 phase	9.5	5.38	0.85	61.65
4 - 2 phase	10	4.96	0.89	79.06
50 h results				
Flask No.	OD550	Glucose g/L	pHS Aq mg/L	pHS Org mg/L
1 - 1 phase	nt	4.97	15.06	na
2 - 1 phase	nt	4.57	16.00	na
3 - 2 phase	nt	0.00	0.28	102.59
4 - 2 phase	nt	0.01	0.28	96.24
Summary				
Flask No.	glucose utilization, g	pHS total mg	---	---
1 - 1 phase	0.63	15.06	---	---
2 - 1 phase	0.65	16.00	---	---
3 - 2 phase	0.88	41.32	---	---
4 - 2 phase	0.88	38.78	---	---

¹nt means not tested

²na means not applicable

EXAMPLE 4

Production of pHS in a Two-Phase Extractive Fermentation System Using 1-Undecanol as Solvent

5 The purpose of this Example was to demonstrate the production of
pHS in a two-phase extractive fermentation system using 1-undecanol as
solvent.

10 The strain used in this study was *E. coli* WWQ51.1, as described in
Example 3. The seed medium used was the same as that described in
Example 3. The test medium was the same as that described in
Example 3, except that lactose was substituted for glucose and IPTG was
added as shown in Table 4. Aliquots of the test medium (50 mL) were
inoculated with a seed culture, 20 mL of 1-undecanol was added to each
flask, and the cultures were incubated at 35 °C and 180 rpm for 48 h in an
incubator shaker. Cultures without 1-undecanol served as controls. After
15 this time, both the aqueous and the organic phases were sampled and
analyzed for pHS using HPLC, as described *supra*. The results are given
in Table 4.

20 Table 4
Results of the Two-Phase Extractive Fermentation
of pHS Using 1-Undecanol as Solvent

Medium mL	IPTG mM	1-undecanol mL	pHS Aq mg/L	pHS Org mg/L	pHS/flask mg
50	0	20	16	1425	29
50	2.5	20	15	1626	33
50	5	20	10	768	16
50	0	0	500	na	25
50	2.5	0	538	na	27
50	5	0	490	na	20

25 These results demonstrate that the pHS produced was extracted
into the 1-undecanol organic phase and that the addition of IPTG had no
significant effect on the pHS yield.

EXAMPLE 5

Comparison of Organic Solvents in the Two-Phase Extractive Fermentation of pHS

The purpose of this Example was to compare the organic solvents DBE obtained from E. I. Du Pont de Nemours and Co. (Wilmington, DE), 2-undecanone, and 1-undecanol in the extractive fermentation of pHS.

The strain used in this study was *E. coli* WWQ51.1, as described in Example 3. The seed medium used was the same as that described in Example 3. The test medium used was the same as that described in Example 3, except that the glucose concentration was lowered to 10 g/L. Aliquots of the test medium (50 mL) were inoculated with a seed culture, 20 mL of the solvent to be tested was added to the appropriate flask, and the cultures were incubated at 35 °C and 300 rpm for 15 h in an incubator shaker. After this time, both the aqueous and the organic phases were sampled. The OD550, pH, glucose concentration and the pHS concentration of the aqueous phases were measured as described in Example 3. The organic phases were analyzed for pHS using HPLC, as described *supra*. The results are given in Table 5.

Table 5
Results of the Two-Phase Extractive Fermentations
of pHS Using Various Solvents

DBE mL	2- undeca- none, mL	1- undeca- nol, mL	OD 550	Glu- cose g/L	pH	pHS Aq mg/L	pHS Org mg/L	Total pHS mg/L	pHS Partition coeff
20	0	0	7.74	0.000	6.52	5	140	61	30
20	0	0	10.48	0.010	6.49	5	145	63	31
20	0	0	8.61	0.006	6.5	5	138	60	29
20	0	0	7.51	0.010	6.54	5	140	61	30
0	20	0	9.39	0.009	6.57	7	140	63	20
0	20	0	9.76	0.007	6.59	6	134	60	23
0	0	20	13.15	1.980	6.5	26	58	49	2
0	0	20	12.53	1.120	6.59	15	81	48	5

These results demonstrate that pHS was extracted into all three of the organic solvents tested. However, the partition coefficient for the extraction of pHS into 1-undecanol was lower than that for the other two

solvents. Consequently, the amount of pHS extracted into the organic phase was higher with DBE and 2-undecanone than with 1-undecanol. Similarly, the total amount of pHS produced was higher with DBE and 2-undecanone as solvents.

5

EXAMPLE 6

Production of pHS Using Two-Phase Extractive Fermentation in a 10 L Fermentor

The purpose of this Example was to demonstrate the production of pHS using two-phase extractive fermentation in a 10 L fermentor with 2-undecanone as solvent.

The pre-seed culture was *E. coli* strain WWQ51.1, as described in Example 3, frozen in glycerol. This culture was used to inoculate 500 mL of the seed medium consisting of KH_2PO_4 (0.15 g/L), Na_2HPO_4 (0.35 g/L), $(\text{NH}_4)_2\text{SO}_4$ (3 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.3 g/L), yeast extract (0.5 g/L), and MOPS (15.7 g/L). The medium was adjusted to pH 6.8 and steam sterilized. Then, glucose (10 g/L), kanamycin (50 mg/L) and ampicillin (100 mg/L) were aseptically added to give the final concentration given in parentheses. The seed culture was inoculated and grown in a 2 L flask incubated at 35 °C and 300 rpm for 12 h in an incubator shaker.

The fermentor medium consisted of: 6.2 L of H_2O , 1.4 g KH_2PO_4 , 3.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7 g $(\text{NH}_4)_2\text{SO}_4$, 7 mL of Mazu DF204 Defoamer, obtained from BASF Corp. (Mt. Olive, NJ), 7 mg thiamine, and 3 L of 2-undecanone, obtained from Bedoukian Research, Inc. (Danbury, CT). After sterilization of the medium, 250 g of a 50% w/v glucose solution and 140 mL of the trace element solution (as described in Example 1), were aseptically added. The pH of the medium was adjusted to 6.5. The final total volume of the fermentation medium was 10 L.

The seed culture was used to inoculate the fermentor medium in a 14 L Braun Fermentor, Biostat C. B. (Braun Biotech International, Melesungen, Germany). The fermentation conditions were as follows: temperature: 35 °C, stir rate: 400-600 rpm, airflow: 2-16 SLPM, DO_2 : 25%, and pressure: 1 bar. Glucose was fed into the fermentor starting at a rate of 0.28 g/min. This rate was reduced when the glucose concentration rose above 2 g/L. IPTG (8 mL of a 0.5 M solution) was added when the glucose concentration fell below 4 g/L.

Samples of the aqueous and organic phases were collected at various times. The glucose, pHCA, CA, phenylalanine, tyrosine, and pHS concentrations, and the OD550 in the aqueous phase were determined,

as described *supra*. The pHS concentration in the organic phase was measured using HPLC, as described *supra*. The results are shown in Table 6.

5

Table 6

The Kinetics of Cell Growth and Fermentation of Glucose to pHCA, CA, Phenylalanine, Tyrosine, and pHS in a Two-Phase Fermentation

Time, h	OD550	Glucose, g/L	pHCA, g/L	CA, g/L	Phe, g/L	Tyr, g/L	pHS Aq, g/L	pHS Org, g/L	Partition coeff.
3.3	3.26	16.1	0.00	0.00	0.02	0.00	nt ¹	nt	nt
6.8	7.25	14.2	0.00	0.01	0.10	0.01	nt	nt	nt
8.6	10.9	8.8	0.00	0.01	0.45	0.05	nt	nt	nt
11.1	14.9	4.9	0.00	0.01	0.59	0.08	nt	nt	nt
13.5	15.4	1.9	0.00	0.06	1.12	0.17	0.004	0.077	19
15.9	18	0.5	0.00	0.18	1.40	0.28	0.007	0.095	14
18.3	20.2	0.0	0.00	0.35	1.15	0.29	0.012	0.239	19
20.2	20.2	0.0	0.00	0.50	1.49	0.37	0.014	0.365	27
23.3	21.8	0.0	0.00	0.70	1.19	0.37	0.012	0.411	33
25.5	21.8	0.0	0.01	0.85	1.31	0.42	0.012	0.559	45
27.3	22.6	0.0	0.01	0.80	1.32	0.45	0.016	0.665	40
33.6	18	0.0	0.01	1.14	1.13	0.47	0.045	0.868	19
36.8	18	0.1	0.01	1.16	1.11	0.48	0.055	1.030	19
39.3	20.8	0.0	0.01	1.19	1.23	0.52	0.055	1.164	21
57.1	18.3	5.5	0.01	1.57	0.82	0.44	0.090	1.570	17
59.4	18.1	7.5	0.01	1.58	0.84	0.44	0.082	1.769	22
63.8	17	7.0	0.01	1.75	0.81	0.42	0.064	1.753	27

¹nt means not tested

10

These results demonstrate the successful production of pHS in a two-phase extractive fermentation in a 10 L fermentor using 2-undecanone as solvent.

EXAMPLE 7

15

Production of Cinnamic Acid (CA) in a Two-Phase Extractive Fermentation System

The purpose of this example was to demonstrate the production of cinnamic acid in a two-phase extractive fermentation system. In addition,

the effect of IPTG, the solvents 2-undecanone and 1-undecanol, and the addition of pHS were investigated.

The strain used in this study was *E. coli* WWQ51.1, as described in Example 3. The conditions were similar to those described in Example 3.

5 Eight test cultures were inoculated; six contained 20 mL of 2-undecanone, the other two contained 20 mL of 1-undecanol. A 10% w/v solution of pHS in propylene glycol (0.5 mL), obtained from Sigma-Aldrich, Milwaukee, WI, was added to two of the cultures containing 2-undecanone, as specified in Table 7.

10 The cultures were incubated in 250 mL baffled flasks at 35 °C and 300 rpm for 24 h in an incubator shaker. After this time, a sample of the aqueous phase was withdrawn and the pH, OD550, and the concentration of the fermentation products were measured, as described *supra*. The organic phase was also sampled and the concentration of CA and pHS
15 were determined using HPLC, as described *supra*.

The results are given in Tables 7, 8, and 9. The data in Tables 7 and 8 demonstrates that *E. coli* can grow in the presence of an inhibitory concentration of pHS in a two-phase fermentation system with 2-undecanone as solvent. Additionally, the addition of IPTG had a
20 significant effect on the amount of CA produced. The results obtained with 1-undecanol were similar to those obtained with 2-undecanone as solvent.

In this study, the pH decreased to low values and a significant amount of CA was extracted into both organic solvents tested. The amount of pHS produced was low, probably because of its poor stability at
25 low pH. As shown by the data in Table 9, the amount of CA extracted into the organic phase was very dependent on the pH of the medium. This pH dependence results because the fully protonated species is the form extracted into the organic phase. The ionized acid form is very water-soluble and is not extracted into the organic phase. Therefore, the
30 extractive fermentation of acidic compounds such as CA and pHCA is favored at low pH, where the acid exists primarily in the unionized form.

Table 7
Effect of Added pHS and IPTG on the Growth and Glucose Utilization
of *E. coli* in Two-Phase Fermentations

Flask No.	IPTG, mM	Solvent added	pHS (10% w/v) added	OD550 0 h	Glucose g/L, 0 h	OD550 24 h	Glucose g/L, 24 h	pH, 24 h
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1	0	2-Undecanone	None	0.13	15.2	15.1	0.0	5.38
2	0	2-Undecanone	None	0.13	15.2	12.1	2.7	4.5
3	0.5	2-Undecanone	None	0.13	15.2	12.9	0.0	5.5
4	0.5	2-Undecanone	None	0.13	15.2	12.3	0.0	5.4
5	0.5	2-Undecanone	0.5 mL	0.13	15.2	15.7	0.0	4.8
6	0.5	2-Undecanone	0.5 mL	0.13	15.2	12.5	0.0	5.7
7	0.5	1-Undecanol	None	0.13	15.2	15.3	0.0	5.7
8	0.5	1-Undecanol	None	0.13	15.2	15.3	0.0	5.71

Table 8
Concentration of Fermentation Products in Two-Phase Fermentations

Flask No.	Results in aqueous phase					Results in organic phase		
	Tyr mg/L	Phe mg/L	pHCA mg/L	CA mg/L	pHS mg/L	pHCA mg/L	CA mg/L	pHS, mg/L
1	347	1087	3	11	nd ¹	<1	39	nd
2	137	796	<1	2	nd	<1	34	nd
3	376	951	22	132	nd	1	189	nd
4	371	942	14	90	nd	1	229	nd
5	268	826	5	26	nd	1	210	3411
6	350	938	21	137	nd	1	151	3966
7	431	1008	16	84	nd	3	176	46
8	428	990	17	88	nd	3	189	33

5 ¹nd means not detected

Table 9
Results of the Two-Phase Extractive Fermentation of CA

Flask No.	Solvent added 20 mL/flask	pH	CA Aq, mg/L	CA Org, mg/L	CA Org/CA Aq
1	2-undecanone	5.38	11	39	3.55
2	2-undecanone	4.5	2	34	17.00
3	2-undecanone	5.5	132	189	1.43
4	2-undecanone	5.4	90	229	2.54
5	2-undecanone	4.8	26	210	8.08
6	2-undecanone	5.7	137	151	1.10
7	1-undecanol	5.7	84	176	2.10

8	1-undecanol	5.71	88	189	2.15
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EXAMPLE 8

Toxicity Testing of Organic Solvents Toward *E. coli*

The purpose of this Example was to screen other organic solvents for toxicity toward *E. coli* using agar cultures.

This study was done using *E. coli* TY1 (DGL430), as described in Example 1. A stock culture of this strain was prepared in 50% glycerol and stored at -20 °C. This stock culture (10 µL) was used to inoculate a 14 mL Falcon™ tube (BD Biosciences, Bedford, MA) containing 5 mL of LB medium, consisting of Bacto-tryptone (10 g/L), Bacto-yeast extract (5 g/L), and NaCL (10 g/L) adjusted to pH 7.5 with NaOH, and supplemented with tetracycline (10 µg /mL). This culture was incubated at 34 °C and 250 rpm overnight in an incubator shaker until an OD600 of between 1.0 and 2.0 was obtained. Dilutions of this culture were spread onto LB agar plates. The appropriate dilution was determined by experimentation to obtain between 100 to 200 colonies per plate. Three plates were used as controls and the remaining plates were used to test the solvents listed in Table 10. The solvents (5 mL) were filtered through a 0.2 µm filter onto the inoculated agar plates. All the solvents were tested in triplicate.

Table 10
Organic Solvents Used in Toxicity Testing

Solvent Name	CAS No.	Source	Purity
Diisopentyl ether	544-01-4	Sigma-Aldrich	99%
Methyl decanoate	110-42-9	Sigma-Aldrich	99%
Propyl Benzoate	2315-68-6	Sigma-Aldrich	99%
Dibenzyl ether	103-50-4	Sigma-Aldrich	99%
2-Decanone	693-54-9	Sigma-Aldrich	99%
1-Phenyl-1-pentanone	1009-14-9	Sigma-Aldrich	99%
2-Tridecanone	593-08-8	Sigma-Aldrich	99%
2-Undecanone	112-12-9	Sigma-Aldrich	97+%
DBE	71195-64-7 141-04-8	E.I. du Pont de Nemours and	98.5%

	925-06-4	Co.	
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The plates were incubated overnight at 30 °C in a vented incubator located in a chemical fume hood. The colonies formed were counted after 24 and 48 h. The colony forming units (CFUs) obtained with each solvent were compared to those of the control plates and plotted in Figure 1 as a percentage versus the log P value for the solvent (P values obtained from Hansch et al., *Exploring QSAR: Hydrophobic, Electronic and Steric Constants*, American Chemical Society, Washington, DC, 1995). The raw data for Figure 1 is given in Table 11. As can be seen from the data in Figure 1 and Table 11, there is a general pattern of decreasing toxicity toward *E. coli* with increasing log P values. The error bars in the figure represent the range of the data. Cell growth on the agar medium was characterized by the presence of both distinct and non-distinct colony formations, which affected the precision of the colony counts. In the presence of some of the solvents tested, growth continued between 24 and 48 h, while with other solvents, no further growth was observed after 24 h. From these results, three solvents were identified as being relatively nontoxic to *E. coli*, 2-tridecanone, methyl decanoate, and DBE, all having a log P value above 3.7. The solvent 2-undecanone was found to significantly inhibit growth in this study, possibly due to impurities in the lot used.

Table 11
Raw Data for the Toxicity Testing Results Shown in Figure 1

Solvent	Log P	% CFU Sample 1	% CFU 1 Sample 2	% CFU Sample 3
DBE	3.84	65	99	35
Methyl decanoate	4.41	73	109	44
1-Phenyl-1-pentanone	3.33	33	62	0
2-Decanone	3.39	0	0	0
Dibenzyl ether	3.1	1.5	5	0
2-Tridecanone	4.95	100	115	87
2-Undecanone	3.91	11	11	11
Propyl benzoate	3.01	9	26	0.9
Diisopentyl ether	3.96	13	39	0

EXAMPLE 9

The Effect of pHS on the Growth of *Pseudomonas putida*

The purpose of this Example was to determine the toxicity of pHS toward *Pseudomonas putida*.

5 This study was done using *P. putida* strain S12 (ATCC No. 700801), obtained from the American Type Culture Collection. This *P. putida* strain was grown at 30 °C in 20 mL of LB medium containing 0.25% glucose and 10 mM MgSO₄ in a 125 mL baffled flask until stationary phase was achieved. Aliquots from this stationary phase culture
10 were plated on LB agar plates containing 0.25% glucose, 10 mM MgSO₄, and 1 g/L pHS, and incubated at 30 °C for several days. Colonies from these plates were streaked on identical plates and grown as described above. An individual colony from these plates was used to inoculate 20 mL of liquid LB medium containing 0.5% glucose, 10 mM MgSO₄ and
15 1 g/L pHS. This culture was grown in a 125 mL baffled flask at 30 °C and 200 rpm in an incubator shaker until stationary phase was achieved. These stationary phase cells (0.5 mL) were added to 20 mL of a test medium consisting of LB medium, 0.5% glucose, 10 mM MgSO₄ and either 0, 1.0, or 1.25 g/L pHS. These cultures were grown in 125 mL
20 baffled flasks at 30 °C and 200 rpm in an incubator shaker. Cell growth in these cultures was monitored by taking aliquots, diluting them with an equal volume of 1,2-propanediol, and measuring the OD at 600 nm. These OD measurements were used to calculate the doubling time and growth rate as a percentage of growth observed in the test medium
25 without pHS. The results from this study are given in Table 12.

Table 12

Growth of *P. putida* in the Presence of pHS

pHS, g/L	Doubling Time, min	Growth Rate, %
0	99	100
1.0	118	85
1.25	111	90

30

These results demonstrate that *P. putida* can be adapted to be tolerant to pHS. Comparison of these results to those given in Example 1, shows that *P. putida* is much more tolerant to pHS than *E. coli* because

the growth of the *E. coli* strain tested in Example 1 was totally inhibited at a pHSA concentration of 0.4 g/L

EXAMPLE 10

The Effect of pHCA on the Growth of *Pseudomonas putida*

5 The purpose of this Example was to determine the toxicity of pHCA toward *Pseudomonas putida*.

 This study was done using *P. putida* strain KT2440 (obtained from Juan Ramos at Consejo Superior de Investigaciones Cientificas, Granada, Spain and *P. putida* strain NCIMB 9866 (obtained from the National
10 Collections of Industrial, Food and Marine Bacteria Ltd., Scotland, UK). *P. putida* strain KT2440 is also available from the American Type Culture Collection as ATCC No. 47054). Liquid cultures of these strains were grown in 10 mL of LB medium containing 0.25% glucose and 10 mM MgSO₄ in 50 mL screw-capped, glass tubes at 30 °C and 200 rpm in an
15 incubator shaker. In all subsequent media, pHCA was added from a 60 g/L stock solution, which was adjusted to pH 7.0 using KOH and then filter-sterilized. Samples of each culture were streaked on LB agar plates containing 0.25% glucose, 10 mM MgSO₄ and 30 g/L pHCA and grown at 30 °C. Individual colonies taken from these plates were grown to
20 stationary phase at 30 °C in 10 mL of LB medium containing 0.5% glucose, 10 mM MgSO₄ and 25 g/L pHCA in 50 mL screw-capped, glass tubes. These stationary phase cultures were diluted 1:25 in fresh LB medium containing 0.5% glucose, 10 mM MgSO₄ and either 0 or 25 g/L pHCA and grown in 50 mL screw-capped, glass tubes at 30 °C and
25 200 rpm in an incubator shaker. Cell growth in these cultures was monitored by taking aliquots and measuring the OD at 600 nm of the culture or dilutions of the culture, which were made with LB medium. These OD measurements were used to calculate the doubling time and growth rate as a percentage of growth observed in test medium without
30 pHCA. The results from this study are given in Table 13.

Table 13
Growth of *Pseudomonas putida* Strains in the Presence of pHCA

Strain	pHCA, g/L	Doubling Time, min	Growth Rate, %
<i>P. putida</i> NCIMB 9866	0	19	100
<i>P. putida</i> NCIMB 9866	25	105	18
<i>P. putida</i> KT2440	0	17	100
<i>P. putida</i> KT2440	25	79	21

- 5 These results demonstrate that *Pseudomonas putida* is more tolerant to pHCA than the *E. coli* strain tested in Example 1 (see Table 1), which was significantly inhibited at a pHCA concentration of 8 g/L.